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Abundance and Diversity of Soybean-Nodulating Rhizobia in Black Soil Are Impacted by Land Use and Crop Management

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To investigate the effects of land use and crop management on soybean rhizobial communities, 280 nodule isolates were trapped from 7 fields with different land use and culture histories. Besides the known *Bradyrhizobium japonicum*, three novel genospecies were isolated from these fields. Grassland (GL) maintained a higher diversity of soybean bradyrhizobia than the other cultivation systems. Two genospecies (*Bradyrhizobium* spp. I and III) were distributed widely in all treatments, while *Bradyrhizobium* sp. II was found only in GL treatment. Cultivation with soybeans increased the rhizobial abundance and diversity, except for the soybean monoculture (S-S) treatment. In monoculture systems, soybeans favored *Bradyrhizobium* sp. I, while maize and wheat favored *Bradyrhizobium* sp. III. Fertilization decreased the rhizobial diversity indexes but did not change the species composition. The organic carbon (OC) and available phosphorus (AP) contents and pH were the main soil parameters positively correlated with the distribution of *Bradyrhizobium* spp. I and II and *Bradyrhizobium japonicum* and negatively correlated with *Bradyrhizobium* sp. III. These results revealed that different land uses and crop management could not only alter the diversity and abundance of soybean rhizobia, but also change interactions between rhizobia and legume or nonlegume plants, which offered novel information about the biogeography of rhizobia.

As a collective name for the symbiotic nitrogen-fixing bacteria associated with legumes, rhizobia cover more than 98 species of the nodule-forming bacteria in 14 genera belonging to the *Alpha-* and *Betaproteobacteria*, like *Rhizobium* and *Burkholderia* (1). These bacteria are very important in ecology and in the economy for their great nitrogen-fixing capability inside nodules, the symbiotic organ induced by rhizobia on their host legume plants. The existence of diverse rhizobia helped the host legumes to adapt to many different habitats (2), while the great diversity and vast geographic distribution of the legumes also shaped their distinct rhizobial populations and drove their diversification (3–5). Therefore, the diversity of rhizobia present in a certain ecosystem is the result of interactions among the rhizobia, their host legumes, and the biotic and abiotic factors of the ecosystem, as revealed by the previous biogeographic studies on the rhizobia associated with faba beans (6), *Caragana* spp. (7), and *Lespedeza* spp. (8).

For free-living bacteria, soil pH was believed to be the determining factor of their biogeography (9). As to the rhizobia, soil pH and salinity are the main ecological factors determining their distribution (10, 11), while agricultural practices, such as crop management (12), tillage intensity (13–16), fertilization (17), legume cultivation history (18, 19), and land use patterns (3), can also modify rhizobial diversity and abundance.

Soybeans are one of the most important legume crops, and extensive studies have been performed on their rhizobia. In general, diverse rhizobia belonging to *Bradyrhizobium* and *Sinorhizobium* are associated with soybeans, and typically, biogeographic patterns have been found among the soybean rhizobia (11, 20). The distribution, abundance, and nitrogen-fixing efficiency of soybean rhizobia are strongly related to genotypes or cultivars of soybeans (21), the latitude (22), and soil environmental conditions, like the organic carbon content, pH, available phosphorus, and other factors (23–25). The dominant soybean rhizobia are *Sinorhizobium fredii* in alkaline-saline soils (11, 20, 22, 26), *Bradyrhizobium liaoningense* in alkaline soils (26), and *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* in acid soils in China (11, 20, 27). However, information about the effects of long-term land use and crop management on the abundance and diversity of soybean rhizobia is limited.

Heilongjiang Province in northeast China is a traditional area for soybean culture. The black soil in that vast area is very fertile and productive with grass marshland vegetation, but the physicochemical properties of the soil were changed drastically after it was reclaimed as agricultural soil about 100 years ago (28, 29). However, many efforts to restore the black soil system, such as restoring the grass vegetation, have been applied to recover the fertility of the ecoregion (29, 30). In relation to the changes in soil properties, a succession of soil bacteria, including the soybean rhizobia, should be expected. Considering the importance of rhizobia for soybean production and the shortage of information about the shifting of soybean rhizobium populations in the agricultural exploration of the black soil, we performed this study. The aim of the current study was to assess the effects of land use and crop man-

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agement on the abundance and diversity of soybean rhizobia in black soil.

MATERIALS AND METHODS

Study site and soil sampling. The study was conducted on experimental fields located in Hailun National Field Station, Chinese Academy of Sciences (longitude 126°38′E, latitude 47°26′N; altitude, 240 m). The experimental region represents typical soil and climate conditions of northeastern China, where it was the main soybean production area. As it is in a continental monsoon climate zone, the region is dry and cold in winter and warm and humid in summer. The experimental region has an average annual rainfall of 500 to 600 mm, 60% of which falls during July and September. The soil is generally classified as black soil (mollisol in the American soil classification system and black chernozem in the Canadian soil classification system) derived from loam loess with approximately 30% clay content (31).

The soil samples were collected from the following plots: (i) Grassland (GL), which was formerly cultivated but returned to natural vegetation since 1985, and now the area has been successfully converted to grassland with *Leymus chinesisas*, *Thalictrum aquilegifoium*, *Vicia pseudo-orobus*, *Calamagrostis epigejos*, *Artemisia japonica*, and other wild plant species; (ii) bare land (BL), which is the same as GL, but the grasses were manually eliminated periodically during the growing season so that there is limited vegetation cover on the site, which was aimed at simulating the process of black soil degradation; and (iii) cropland (CL), which had been continuously cultivated with maize (*Zea mays* L. cv. Haiyu 6)/soybean [*Glycine max* (Merrill) L. cv. Heinong 35]/wheat (*Triticum aestivum* L. cv. Long 4083) without a fertilizer supply since 1985. The CL plot was subdivided into five subplots in 1990 for different types of agricultural management. One of the subplots was maintained under maize/soybean/wheat rotation without fertilizer supply (CL), and the other subplots were cultivated under soybean, maize, and wheat monoculture (S-S, M-M, and W-W) and maize/soybean/wheat rotation (M/S/W), with conventional agricultural management, including fertilization and tillage. The fertilizer applications (per hectare) were as follows: (i) 112.5 kg N, 45 kg phosphorus pentoxide (P_2O_5), and 30 kg potassium oxide (K_2O) for maize; (ii) 20.25 kg N, 51.75 kg P_2O_5, and 30 kg K_2O for soybeans; and (iii) 112.5 kg N, 45 kg P_2O_5, and 30 kg K_2O for wheat. The areas of the GL and BL plots were about 360 m^2 and 180 m^2, respectively, and the CL plot was 60 m^2 with four replicates. The experiment area had never received any rhizobial inoculants.

After wheat was harvested in July 2013, three soil samples were recovered from each plot of the 7 treatments. Each of the soil samples consisted of soils from 5 randomly selected sites within each plot at a depth of 0 to 20 cm mixed in a sterilized bag. Soil samples were collected with a soil drill cleaned with ethanol (95%) and flamed before each sampling.

Soil samples were divided into two parts. One part was first air-dried and finely ground to pass through 2-mm mesh screens and then used to determine the basic properties, like the moisture content after drying the soil at 105°C for 4 days. The soil pH was measured in water suspension (H_2O/soil ratio, 2.5:1) (32). The organic carbon (OC) content of the soil was determined using a wet-oxidation method with $K_2Cr_2O_7$ and concentrated H_2SO_4 (33). The soil available nitrogen (AN) content was analyzed by quantifying the alkali-hydrolyzable N in a Conway diffusion unit with Devarda's alloy in the outer chamber and boric acid-indicator solution in the inner chamber (34). The available phosphorus (AP) content was measured by means of colorimetry after extraction with 0.5 mol $liter^{-1}$ $NaHCO_3$ (pH 8.5) for 30 min (35, 36). The available potassium (AK) content was measured with a flame photometer after extraction with 1 mol $liter^{-1}$ NH_4Ac (pH 7.0) for 15 min (36). The soil bulk density (BD) was calculated based on the inner diameter of the core sampler's cutting edge, and the soil was oven dried at 105°C for 12 h (37). Another part of the soil samples was stored at 4°C and used within 2 weeks for rhizobium counting and isolation by plant trapping.

Rhizobiun count and isolation. For both the evaluation of the rhizobium population in soil and trapping of rhizobia, three surface-sterilized soybean seeds of cultivar Heinong 35 (a popular cultivar in northeast China) were sown in a Lenard jar filled with sterile vermiculite (38). After 3 days of germination, two seedlings were left per jar, and 1 ml of soil gradient dilution (from 10^{-1} to 10^{-9}; 5 replicates for each dilution) was inoculated into the root zone by injection, as reported previously (38). The plants were grown under greenhouse conditions, with temperatures of 28 and 22°C (day/night), and received nitrogen-free nutrient solution every 2 or 3 days. Plants were harvested 5 weeks after emergence, and the roots were washed to evaluate nodulation. The rhizobial abundance was evaluated by the most probable number (MPN) counting technique (38) and was expressed as the number of cells gram dry $soil^{-1}$. For each treatment, 40 nodules were randomly collected from the plants inoculated with a dilution of 10^{-2}. The collected nodules were surface sterilized with 95% ethanol for 30 s and 2.5% hydrogen peroxide for 3 min. After being rinsed 7 times using sterilized distilled water, the nodules were crushed separately in 1.5-ml sterilized microtubes. The nodule juice was stroked on plates of yeast-mannitol agar (YMA) (38), which were incubated at 28°C for 1 to 2 weeks for isolation of the rhizobia. The bacterial colonies obtained were purified by repeated streaking on YMA medium. Pure cultures were maintained on YMA slants at 4°C for short-term storage or in YM broth supplied with 20% (wt/vol) glycerol at −80°C for long-term storage.

PCR amplification and sequencing analysis of housekeeping and symbiotic genes. Genomic DNA was extracted from each isolate using the GUTC method (39). Primers recA41F and recA640R and PCR procedures were used to amplify the *recA* gene (40). The PCR products were sequenced directly (41). All the *recA* sequences acquired in this study were used to determine the phylogenetic positions of the isolates with Clustal W software (42). Isolates sharing identical sequences were designated a single genotype.

Based upon the grouping results in *recA* gene analysis, representative isolates of different genotypes were chosen to obtain the other housekeeping genes. The other housekeeping genes, *glnII*, *atpD*, *dnaK*, *gryB*, and *rpoB*, were amplified using primer pairs glnII12F/glnII689R, atpD255F/atpD782R, TsdnaK3/TsdnaK2, gryB343F/gryB1043R, and rpoB454F/rpoB1364R, respectively (40, 43–45). A fragment of the *nifH* gene (about 800 bp) was amplified with the primer pair nifHF/nifHR and the protocol of Laguerre et al. (46). A fragment of the *nodC* gene (about 700 bp) was amplified with primer pair nodCF540/nodCR1160 using the protocol of Sarita et al. (47). The PCR products for each housekeeping gene were sequenced directly, like the *recA* sequencing. The acquired sequences were deposited in the GenBank database. All sequences acquired in this study and homologous sequences obtained from the GenBank database were aligned using the ClustalW software (42). Phylogenetic trees were reconstructed for each gene using the neighbor-joining method (48) with Kimura's two-parameter model and were bootstrapped with 1,000 replications using the MEGA 5.0.5 package (49).

Since multilocus sequence analysis (MLSA) has greater discriminatory power than only one gene (such as *recA* and 16S rRNA), as revealed in studies on *Sinorhizobium* and *Bradyrhizobium* (44, 50), MLSA of the combined housekeeping genes (*recA*, *glnII*, *atpD*, *dnaK*, *gryB*, and *rpoB*) was conducted, and the sequence similarity with related strains was calculated as described above.

Statistical analysis. To estimate the community structure and species richness of soybean rhizobia, genospecies were defined based upon the results of MLSA sequence analysis of the above-mentioned six housekeeping genes in this study. Soybean rhizobial diversity, species richness, and evenness in different treatments were estimated by three popular alpha ecological indexes (51): the Shannon-Wiener index (H'), representing diversity considering the species richness in a community, and the Simpson index (D) and the Pielou index (J), showing the species dominance and evenness, respectively, in a community. These indexes of biodiversity

were implemented in the Vegan package (version 1.17-4) and calculated using the R statistical language (version 3.1.0) (52).

Redundancy analysis (RDA) (53), the canonical version of principal-component analysis (PCA), was used to examine the effects of soil use/crop management on the soil factors (organic carbon, BD, AN, AP, AK, and soil pH) and the diversity of soybean rhizobia in the 7 sampling sites. Community data for rhizobia (Table 1) were preanalyzed by detrended correspondence analysis (DCA) using CANOCO software 4.5 (Microcomputer Power, Ithaca, NY) (70). In the DCA, the models of species response to environmental variables and the length of the gradient (first axis) were 1.123, so both the linear model and the unimodal model are suitable. After further model tests, RDA proved to be the best method.

Nucleotide sequence accession numbers. The sequence data from this study have been deposited in GenBank under accession numbers KJ547727 to KJ547732 (*atpD*), KJ547733 to KJ547738 (*dnaK*), KJ547739 to KJ547744 (*glnII*), KJ547745 to KJ547750 (*gyrB*), KJ547763 to KJ547768 (*rpoB*), KJ547769 to KJ547774 (*recA*), KJ547757 to KJ547762 (*nodC*), and KJ547751 to KJ547756 (*nifH*) for representative isolates of strains M10, G35, C24, B8, S36, and M26.

RESULTS

Soil properties in different treatments. The basic properties of soils are listed in Table 1. In general, all the soils were acid, with pHs varying from 5.68 (lowest) in M-M fields to 6.30 (highest) in GL. Long-term natural restoration of grassland in black soil significantly increased the OC and AP contents and the pH with the order BL < CL < GL (Table 1), and the OC content of GL increased by 19.6% and 40.4%, respectively, compared with CL and BL. In contrast, the BD increased opposite to the OC and AP contents and the pH, showing negative correlation with them. The AK and AN contents were highest under the GL and lowest under the CL treatment due to the crop removed. After 27 years of crop managements with fertilizer applied, the M/S/W treatment had higher OC, AN, AP, and AK contents and higher pH than the M-M, W-W, and S-S treatments. Consequently, the OC content of M/S/W rotation treatment was increased by 6.7%, 10.2%, and 6.7%, respectively, compared to M-M, W-W, and S-S. Additionally, these monoculture treatments did not appear to affect the OC, AN, or AK content and the BD compared to M/S/W rotation. Moreover, due to lack of fertilizer application since 1985, the OC, AN, AP, and AK contents of CL treatment were lower than those of M/S/W treatment.

Population abundance and isolation of soybean rhizobia in different treatments. The abundances of soybean rhizobia under different land use and crop management are shown in Table 1. Briefly, the rhizobial abundances in soils after 27 or 22 years with different land use and crop management showed the order M/S/W > S-S > CL > GL > W-W > BL > M-M. The abundance of soybean rhizobia in CL increased by 0.75- and 72.3-fold, respectively, compared with those in GL and BL. Compared to crop management, the rhizobial abundance in rotation with fertilization (M/S/W) was 15.1, 61.8, 2,518.5, and 11,147.5 times greater than those in S-S, CL, W-W, and M-M.

Diversity and composition of rhizobial populations in different treatments. In the *recA* gene sequence analysis, all the isolates were grouped into 4 genospecies within the genus *Bradyrhizobium*, and they exhibited high sequence similarities (95.2% to 100%) with the known species (see Fig. S1 and Table S1 in the supplemental material). The phylogenetic trees constructed with the housekeeping genes *recA*, *glnII*, *atpD*, *dnaK*, *gryB*, and *rpoB* separately (see Fig. S1 in the supplemental material) showed topology similar to that of the combined sequences of the six genes

TABLE 1 Soil properties, abundance, and genetic diversity of soybean rhizobia in 7 fields with different land use and crop management

Land use and crop management[a]	Characteristics of soil factors[b]						Rhizoidal abundance and genospecies composition [no. (%)][c]				Diversity index		
	pH (H_2O)	OC (g kg^{-1})	AN (mg kg^{-1})	AP (mg kg^{-1})	AK (mg kg^{-1})	BD (g cm^{-3})	MPN (no. of cells g dry $soil^{-1}$)	*Bradyrhizobium* sp. I	*B. japonicum*	*Bradyrhizobium* sp. II	*Bradyrhizobium* sp. III	H'	D	J
GL	6.30a	35.53a	218a	12.20e	183.2a	0.95c	6.3×10^4	17 (42.5)	5 (12.5)	4 (10.0)	14 (35.0)	1.325	0.671	0.956
BL	5.99cd	25.31e	220a	9.40f	153.2c	1.06a	1.5×10^3	6 (15.0)	3 (7.5)	0	31 (77.5)	0.676	0.371	0.616
CL	6.19b	29.70c	179b	11.35e	140.5d	1.03ab	1.1×10^5	20 (50.0)	4 (10.0)	0	16 (40.0)	0.943	0.580	0.859
S-S	5.96d	31.30c	210a	14.10d	164.4bc	0.99bc	4.5×10^5	35 (87.5)	0	0	5 (12.5)	0.377	0.219	0.544
M-M	5.68e	30.30c	212a	15.40c	161.6bc	0.96bc	6.1×10^2	13 (32.5)	0	0	27 (67.5)	0.631	0.439	0.910
W-W	6.01cd	31.30c	216a	16.5b	167.0b	0.98bc	2.7×10^3	15 (37.5)	0	0	25 (62.5)	0.662	0.469	0.954
M/S/W	6.11bc	33.40b	220a	26.5a	171.6b	0.97bc	6.8×10^6	26 (65.0)	10 (25.0)	0	4 (10.0)	0.857	0.505	0.780
Total	–[d]	–	–	–	–	–	–	132 (47.1)	22 (7.9)	4 (1.4)	122 (43.6)	–	–	–

[a] GL, grassland since 1985; BL, bare land since 1985; CL, cropland with maize/soybean/wheat rotation without fertilizer supply since 1985; S-S, soybean monoculture since 1990; M-M, maize monoculture since 1990; W-W, wheat monoculture since 1990; M/S/W, maize/soybean/wheat rotation with chemical fertilizer supply since 1990.

[b] Different letters within columns indicate significance at a P value of <0.05.

[c] Abundance was determined by MPN; the genospecies was identified by multilocus sequence analysis including the genes *recA*, *atpD*, *glnII*, *gryB*, *rpoB*, and *dnaK*.

[d] –, not calculated.

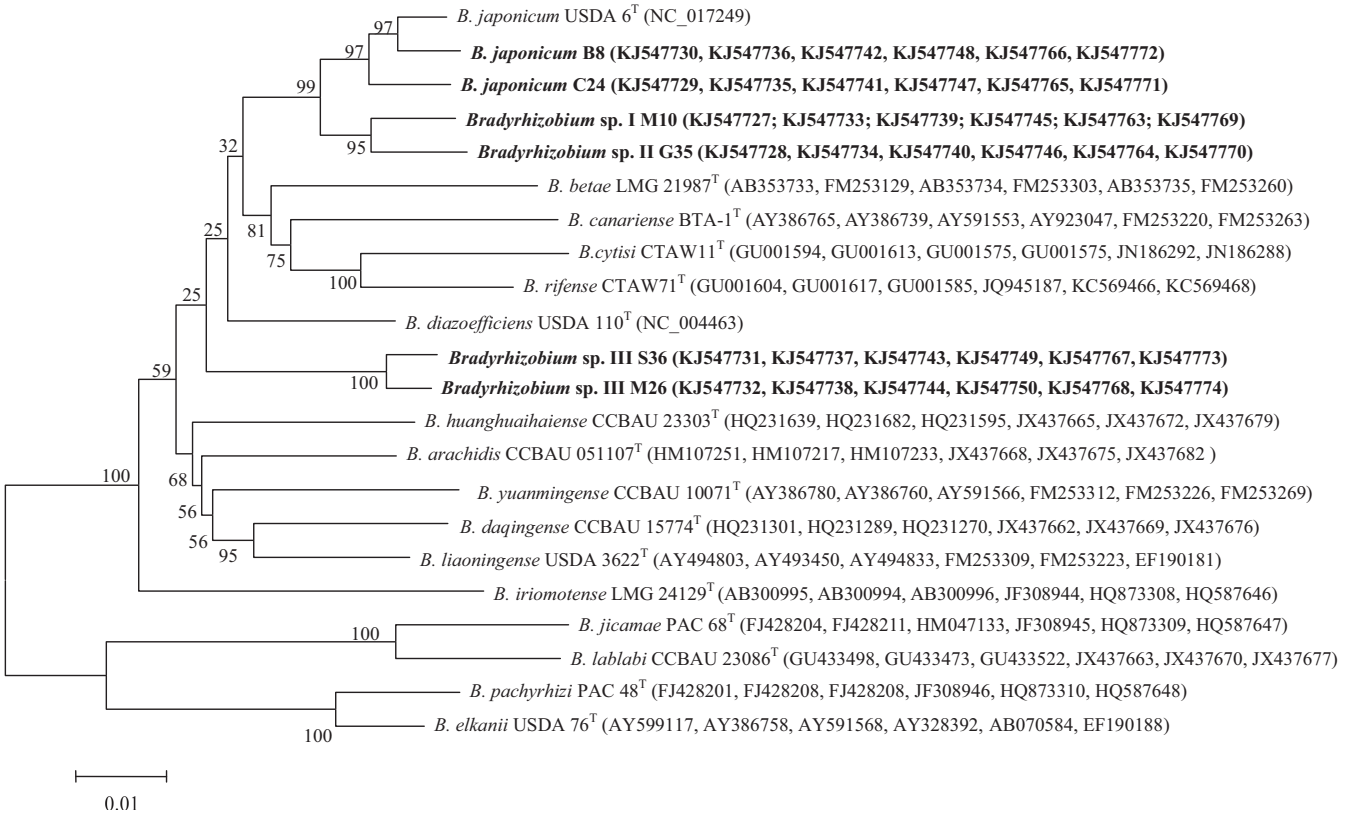

FIG 1 Phylogenetic tree of MLSA based on concatenated sequences of *recA* (457 nucleotides [nt]), *glnII* (512 nt), *gryB* (590 nt), *rpoB* (761 nt), *dnaK* (223 nt), and *atpD* (419 nt). Taxa and GenBank accession numbers in boldface were newly determined as a result of this study. The tree was constructed by the neighbor-joining method. Bootstrap confidence levels of 50% are indicated at the internodes. The bar represents 1% nucleotide divergence.

(Fig. 1). Due to the fact that *B. elkanii* USDA 76^T and *Bradyrhizobium pachyrhizi* PAC 48^T were separated with a threshold value of 97.7% sequence similarity in MLSA of the combined housekeeping genes (*recA*, *glnII*, *atpD*, *dnaK*, *gryB*, and *rpoB*), the value of 97.7% was used as the criterion for species separation of the isolates in the present study. Isolates of B8 and C24, representing 22 isolates, grouped with *B. japonicum* USDA 6^T, sharing 98.3% and 98.8% similarity in MLSA, and were identified as *B. japonicum* (see Tables S1 and S2 in the supplemental material). Isolates of M10 and G35, representing 132 and 4 isolates, respectively, were two lineages closely related to *B. japonicum* USDA 6^T, sharing 97.7% and 96.3% similarity, respectively, in MLSA. They were designated *Bradyrhizobium* spp. I and II, respectively (see Tables S1 and S2 in the supplemental material). Isolates of S36 and M26, representing 122 isolates, were a branch clearly distinct from all the defined species, and they shared 95.5% similarity with *Bradyrhizobium diazoefficiens* USDA 110^T in MLSA analysis (see Tables S1 and S2 in the supplemental material) and were identified as *Bradyrhizobium* sp. III.

Unlike the phylogenetic analysis of the housekeeping genes, the *nodC* and *nifH* genes of the 6 representatives showed 99.6% to 100% sequence similarity to those of *B. japonicum* USDA 6^T, *B. diazoefficiens* USDA 110^T, *Bradyrhizobium daqingense* CCBAU 15774^T, and *Bradyrhizobium huanghuaihaiense* CCBAU 23303^T (Fig. 2; see Fig. S2 in the supplemental material). All were isolated from soybeans.

The compositions of soybean rhizobia in soils with different land use and crop management are listed in Table 1. Briefly, four genospecies were isolated from GL; three genospecies were isolated from BL, CL, and M/S/W; and two genospecies were isolated from S-S, M-M, and W-W. GL and S-S always presented the highest and the lowest diversity of rhizobia, respectively, according to the 3 indexes of diversity (Table 1). In addition, distinct community compositions were found in different treatments, and *Bradyrhizobium* spp. I and III were found as predominant groups in all treatments. The most predominant genospecies were *Bradyrhizobium* sp. III in BL (77.50%), M-M (67.5%), and W-W (62.5%) and *Bradyrhizobium* sp. I in GL (42.5%), CL (50%), S-S (87.5%), and M/S/W (65.0%). As minor groups, *B. japonicum* was found only in GL, BL, CL, and M/S/W, while *Bradyrhizobium* sp. II was found only in GL.

Correlation among the soil properties and distribution of soybean rhizobia. The relationships between soil environmental factors and soybean rhizobial genospecies in northeast China are shown in Fig. 3. According to the lengths of the arrows and the angles among them (Fig. 3), the OC content and pH had a strong positive correlation with the existence of *Bradyrhizobium* sp. I, *B. japonicum*, and *Bradyrhizobium* sp. II and strong negative correlation with the distribution of *Bradyrhizobium* sp. III. The AK and AP contents had slight effects on the distribution of soybean rhizobia, because the arrows representing them are relatively short. Based upon the direction of the arrows, the effects of pH and the AP and AK contents were the same as those of the OC content, and the effects of BD were in contrast to those of the OC content. As

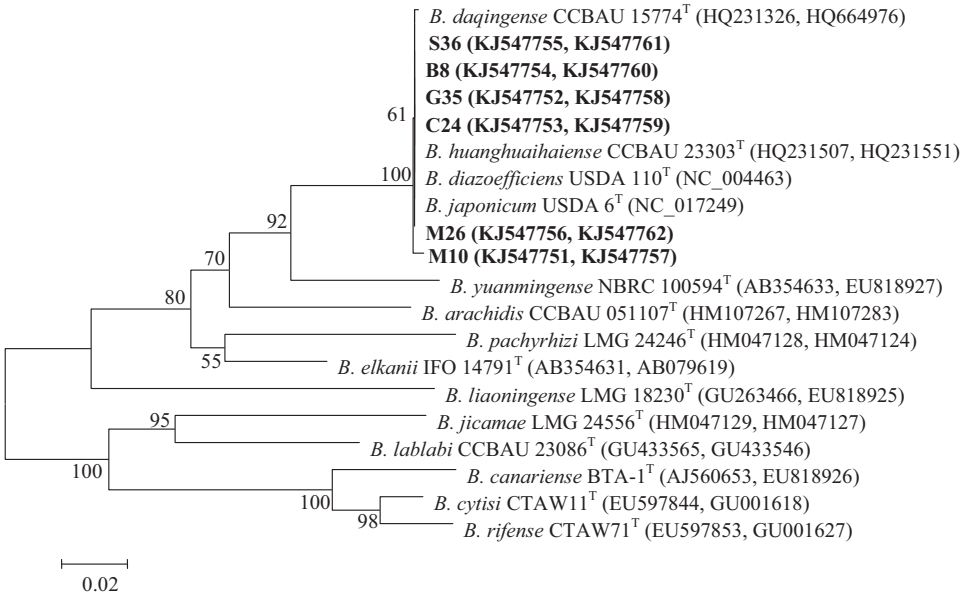

FIG 2 Phylogenetic relationships of the soybean rhizobia isolated from the black soils based on the concatenated sequences of *nodC* and *nifH*. The tree was constructed by the neighbor-joining method. Bootstrap values greater than 50% are marked at the nodes. The scale bar represents 2% nucleotide substitution.

shown in Fig. 3, the BD in soil was positively correlated with the distribution of *Bradyrhizobium* sp. III but negatively correlated with *Bradyrhizobium* sp. I, *B. japonicum*, and *Bradyrhizobium* sp. II and the population size (abundance) of soybean rhizobia in the soil.

DISCUSSION

Unique community structure of soybean rhizobia in black soil. In the present study, we characterized by the MLSA method the rhizobial populations trapped by soybean plants from slightly acid (pH 5.68 to 6.30) black soils rich in OC (25.31 to 35.53 g kg dry soil^{-1}) with different land use and crop management in Heilongjiang Province, northeast China (Table 1). All 280 rhizobial isolates obtained from the 7 soil samples were designated *Bradyrhizobium* spp. (Table 1), which confirmed the previous observations that *Bradyrhizobium* species were the predominant microsymbionts for soybeans in acid and neutral soils (11, 54). However, the identification of *Bradyrhizobium* sp. I and *Bradyrhizobium* sp. III as the dominant groups and *B. japonicum* and *Bradyrhizobium* sp. II as the minor groups in black soils (Table 1 and Fig. 1) revealed a unique community structure different from those reported previously, in which *B. japonicum* and *B. elkanii* were predominant soybean rhizobia in acid and neutral soils and *Sinorhizobium* and *B. liaoningense* in alkaline-saline soils in different regions of China (11, 20, 26, 55). In addition, *B. liaoningense* was the dominant group in alkaline soils in India (27), while the predominance of *B. japonicum* in the northern United States and of *B. elkanii* in the middle to southern United States was described (22). The predominance of *Bradyrhizobium* spp. I and III in this study demonstrated that they were the soybean rhizobia most adapted to the black soils in that region. Therefore, our results in this study have added new evidence for the biogeographic patterns of soybean rhizobia, enlarged the diversity of soybean nodulating rhizobia, and revealed the existence of novel bradyrhizobial species. Since adaptation to environmental stress would result in a high degree

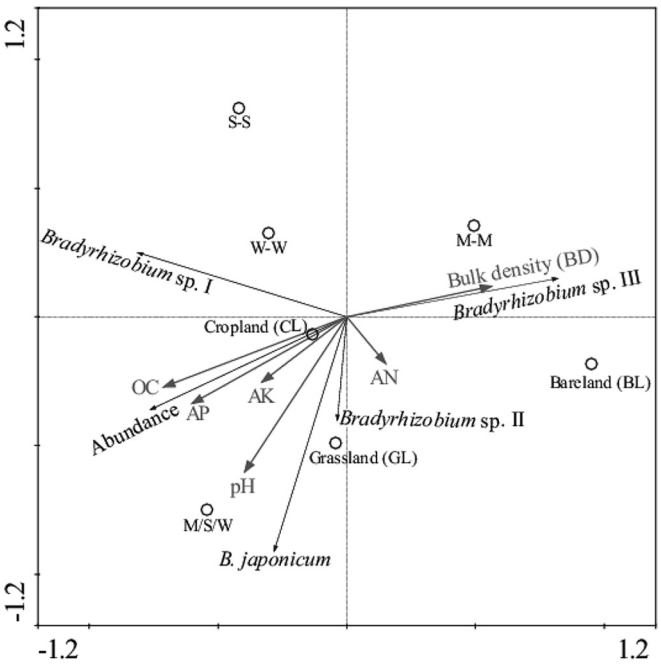

FIG 3 Biplot of the RDA on the 4 genospecies, abundances of rhizobia, and the soil factors from soil samples under different land use and crop management by CANOCO software. The gray arrows represent soil properties. The black arrows show the abundances of the four genospecies isolated from nodules or the abundances of rhizobia in soil calculated by the MPN method. The circles represent different land uses or crop management. The longer the gray arrow, the greater the influence of that specific soil property on the distribution of the genospecies; the smaller the angle between the gray and black arrows, the closer the relationship between the soil factor and the rhizobial genospecies. The distances between the circles reflect their dissimilarity. Projecting a circle onto the black arrow quantifies the relationship between the relative proportion of a specific genospecies and soil use/crop management; the nearer the projection point to the black arrow, the closer their relationship.

of genetic changes of soybean bradyrhizobia (56, 57), the finding of a unique rhizobial community in the black soil not only offered novel information for the biogeography of rhizobia, but also provided novel candidates for evolutionary study of the rhizobia in different environments.

Despite the definition of four genospecies, only one symbiotic type was defined among the isolated bradyrhizobia from the tested soils (Fig. 2). The almost identical *nodC* and *nifH* nucleotide sequences among our isolates and those of *B. japonicum*, which is the main soybean rhizobium in the center of origin for the plant and coexisted with the new groups in the tested soils, implied that these novel genospecies might have obtained their symbiotic genes by lateral gene transfer from *B. japonicum*, similar to the cases in some other reports (54, 58, 59).

Effects of land use and crop management on rhizobial populations. In the present study, the population abundances, community structures, and diversity of effective soybean rhizobia varied drastically among the treatments (Fig. 1 and Table 1), reflecting the fact that the rhizobia were strongly affected by soil management, like the cropping system, land use patterns, and fertilizer input, which were similar in some respects to previous reports (12, 58, 60) and also provided some new information.

Our results supported the observations of Abaidoo et al. (58), who reported that the lower bradyrhizobial populations in farmers' fields could be attributed to the low legume stand densities and fertilizer inputs. In the present study, the culturing of soybeans (CL, S-S, and M/S/W) significantly increased the abundance of rhizobia (1.1×10^5 to 6.8×10^6 cells g dry soil^{-1}) in comparison with those in GL, BL, and M-M or W-W (6.3×10^4 to 6.1×10^2 cells g dry soil^{-1}). The greater abundance of rhizobia in M/S/W than in CL and S-S (Table 1) reflected the positive effects of fertilization and rotation on the rhizobial populations, as reported previously (14, 58). In addition, the higher abundance of rhizobia in GL than in BL, M-M, and W-W demonstrated that the existence of diverse nonlegume plants could help the rhizobia to maintain their abundance at a higher level. Furthermore, the detection of rhizobia in soils where soybeans were absent for 27 years (BL, M-M, and W-W treatments) supported the earlier suggestion that soybean rhizobium populations are quite persistent (14, 61). Thus, the population abundance of rhizobia was determined not only by the host legume cultivation history, but also by fertilizer application and crop management.

Reasons for the variation and diversity of rhizobial populations may be due to the different land use histories, rotation or monoculture of the host plant, different fertilizer supplements, and planting of legume or nonlegume plants. First, the highest rhizobial diversity was obtained from GL treatment, as revealed by the presence of all four genospecies and the three diversity indexes (Table 1), implying that the diverse plant communities are more efficient than the host plant itself in maintaining the diversity of soybean rhizobia. The higher diversity of rhizobia in the rotation systems (CL and M/S/W) than in the monoculture system (S-S) also confirmed this observation and was consistent with previous results for *Rhizobium leguminosarum* (12). Second, the other treatments, except for GL, had lower diversity indexes, and no *Bradyrhizobium* sp. II bacteria were isolated (Table 1), demonstrating that mowing grass or converting grassland to agricultural use decreased the diversity of soybean rhizobia, which was similar to the effects of deforestation on native *Bradyrhizobium* communities (60). Third, fertilization did not change the genospecies composition of soybean rhizobia but decreased the diversity of the rhizobia, as shown by the increased relative proportion of *Bradyrhizobium* sp. I and *B. japonicum*, and decreased that of *Bradyrhizobium* sp. III. in CL treatment in comparison with that of M/S/W. Previously, decrease of genetic diversity caused by fertilization was also found in bean-nodulating rhizobia, but the study was only at the genetic level and did not clarify the genospecies (17). Fourth, monoculture of soybeans and nonlegume plants (such as maize and wheat) decreased the diversity of soybean rhizobia, eliminating *B. japonicum* and *Bradyrhizobium* sp. II while enhancing the relative proportion of the other two rhizobial genospecies. However, the cultivation of soybeans significantly increased the proportion of *Bradyrhizobium* sp. I (87.5%), while maize and wheat selected *Bradyrhizobium* sp. III (67.5% and 62.5%). These data demonstrated that maize and wheat also have specific effects on certain rhizobial species, although they are not symbiotic hosts for the rhizobia. Generally, crop monoculture was known to have allelopathic chemicals that may change soil properties and thus influence the population abundance and diversity of rhizobia (62). Finally, cultivation of soybeans could increase the abundance of rhizobia in CL, S-S, and M/S/W but decrease the diversity of the rhizobial community in comparison with that in M/S/W. All of these observations revealed the complicated interactions among the rhizobia, their host plants, and the biotic and abiotic factors in their habitats.

Certainly, the soil conditions and specific affinity between the rhizobia and host plants can determine the community structure and population abundance of the rhizobia, as revealed in previous reports (11, 20, 22, 26, 54) and in this study (Table 1). However, further studies are needed to discover the mechanisms through which the nonlegume plants can selectively maintain certain rhizobial species and to explain why the increased diversity of nonlegume plant species helped maintain higher diversity of rhizobia in the soil. A possible reason may be that land use or crop management changed the soil conditions and subsequently affected the rhizobial population abundance. For example, mowing grass (BL) significantly reduced the contents of OC, AP, and AK and the pH but increased the BD compared with the GL treatment (Table 1). Another consideration is the possible association of rhizobia as endophytes of the other plants in the grassland and maize/wheat in the cropland, since endophytic *R. leguminosarum* in rice (63) and *Rhizobium etli* in maize (64) have been reported. It is possible that the plants have a preference for their endophytes, which in turn affected the diversity and abundance of rhizobia.

Determinants of the distribution and diversity of rhizobial populations. Similar to the results in previous reports (9), the distribution, population abundance, and diversity of soybean rhizobia are directly influenced, not only by the soil conditions (mainly pH), but also by the host plants (Table 1 and Fig. 3). Generally, conversion from natural ecosystems to artificially regulated ecosystems (e.g., from grassland to arable land) is often accompanied by drastic changes in soil properties (29, 65–67). Our results also demonstrated that mowing and culturing of crops in the fields decreased the pH and OC, AP, and AK contents but increased the soil BD (Table 1). These changes subsequently affected the population abundance, distribution, and diversity of the soybean rhizobia in the soils. The significantly positive correlation between the OC and AP contents, the pH, and the distribution of soybean rhizobia in our study were similar to previous observations (11, 59). Grossman et al. (68) stated that the soil

carbon content may play a role in determining rhizobial communities, as organic farming systems had 15 to 200% more soil carbon than conventional farming systems (68, 69). The identification of soil pH as one of the main ecological factors that determine abundance and diversity in the present study was similar to findings of previous reports for soybean rhizobia (18, 20, 26). In the present study, the close relationship between *B. japonicum* and *Bradyrhizobium* sp. II, their common close correlation with OC and AP contents and pH and weaker relationship with AK and AN might explain why they are eliminated simultaneously in the three monocultures (Table 1). The slight correlation of the AK content with some soybean rhizobial groups was also found by Zhang et al. (11). Meanwhile, *B. japonicum* showed stronger relationships than *Bradyrhizobium* sp. II to the above-mentioned soil parameters, which might explain why *Bradyrhizobium* sp. II was found only in the GL treatments while *B. japonicum* existed in the two rotation treatments (CL and M/S/W). *Bradyrhizobium* sp. II may be more sensitive to the decrease in the OC and AK contents and pH than all the other genospecies (Table 1). The weak relationships of the above-mentioned soil parameters to *Bradyrhizobium* sp. I and their opposite effects on *Bradyrhizobium* sp. III could explain their increased proportions while *B. japonicum* and *Bradyrhizobium* sp. II reduced their proportions. However, the effect of the BD was in contrast to that of the OC content and was positively correlated with the distribution of *Bradyrhizobium* sp. III but negatively correlated with *Bradyrhizobium* sp. I, *B. japonicum*, *Bradyrhizobium* sp. II, and the abundance of soybean rhizobia in the soil. In this case, it was not clear whether the changed bulk density in the studied soils directly affected the survival of rhizobia or if the correlation of BD with the rhizobial distribution was only a reflection of the change in land use.

In conclusion, our present results not only clearly demonstrated that natural vegetation restoration and crop management affect the diversity and abundance of rhizobia, but also uncovered an interesting interaction between the rhizobia and legume and nonlegume plants, which offered novel information for the biogeography of rhizobia, for interactions among the rhizobia and their biotic and abiotic environmental factors, and for the evolution of rhizobial populations adapted to the significant changes in soil properties. It will also be interesting to examine the nodulation matching and nitrogen fixation efficiencies of these different rhizobial genospecies nodulated to soybean cultivar Heinong 35 or other cultivars in monoculture, intercropping, or rotation or in different fertilization systems in future studies.

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